Original Article

Suppressing the activity of *CXCR4* down-regulates the expression of renal fibrosis related genes in primary glomerular cells

Shaofan Hu, Hong Liu, Qun Li, Qiang Yu, Xiaoqing Liu, Jin Xu, Rui Fu

Department of Nephrology, Jiangxi Provincial Children's Hospital, Nanchang, China

Contributions: (I) Conception and design: S Hu, R Fu; (II) Administrative support: R Fu, H Liu; (III) Provision of study materials or patients: H Liu, Q Li; (IV) Collection and assembly of data: Q Yu, X Liu; (V) Data analysis and interpretation: S Hu, J Xu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Rui Fu. Department of Nephrology, Jiangxi Provincial Children's Hospital, No. 1666 Diezihu Avenue, Qingshanhu District, Nanchang, China. Email: ruifu2008@126.com.

Background: C-X-C chemokine receptor type 4 (*CXCR4*) has a certain effect on renal fibrosis, and there are few specific studies in cells. We want to investigate the impact of suppressing *CXCR4* activity on the expression of renal fibrosis-related genes in primary glomerular endothelial cells, mesangial cells, and podocytes.

Methods: Immunofluorescence assays were used to determine the purity of isolated glomerular endothelial cells, mesangial cells, and podocytes. *CXCR4* knockdown cell lines were established by transfecting the short hairpin (sh)RNA against CXCR4. T140 and AMD3100 were used to inhibit the activity of *CXCR4*. LY294002 was used to inhibit the activity of phosphoinositide 3-kinase (*PI3K*). The mRNA expression of *CXCR4* was determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). The protein expression of *CXCR4*, *collagen IV*, matrix metallopeptidase *(MMP)-9*, *PI3K*, *Rac1*, and vascular cell adhesion protein 1 (*VCAM-1*) was evaluated by Western blot analysis.

Results: High purity was observed on isolated primary glomerular endothelial cells and podocytes. However, the purity of isolated mesangial cells was relatively low. The mRNA expression of *CXCR4* was significantly suppressed by the transfection of shRNA. Compared to control cells, the expression of *CXCR4*, collagen *IV*, *MMP-9*, *PI3K*, *Rac1*, and *VCAM-1* were dramatically downregulated in cell lines transfected with shRNA against *CXCR4*. Furthermore, cell lines treated with T140, AMD3100, or LY294002 also showed downregulated expression of these proteins compared to untreated cells. No significant differences were observed in the protein expression of these proteins between control cells and cells transfected with the shRNA negative control (NC).

Conclusions: Suppressing the activity of *CXCR4* downregulated the expression of renal fibrosis-related genes in primary glomerular cells, even under a non-inflammatory state.

Keywords: C-X-C chemokine receptor type 4 (*CXCR4*); renal fibrosis; T140; AMD3100; glomerulus

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Introduction

Renal fibrosis is defined as excessive growth, sclerosis, or scarring of kidney tissue due to excessive accumulation of extracellular matrix components, such as collagens. Severe cases of renal fibrosis may result in renal dysfunction (1). Renal fibrosis can be induced by almost all kinds of chronic renal diseases or injuries, such as sustained infections, autoimmune reactions, anaphylaxis, chemical injuries, radiation, and mechanical injuries (2). Chemokines are peptides with molecular weight ranging from 8 to 12 kDa, and are named for its chemotaxis against leukocytes.

Chemokines have been reported to be involved in the development and progression of fibrosis by recruiting myofibroblasts, macrophages, and other effector cells to the site of injury (3,4). It has been demonstrated that pulmonary fibrosis can be significantly ameliorated by downregulating the expression chemokines, such as *C-C* motif chemokine ligand (*CCL*)*2* and *CCL3*, or by blocking their receptors (5-7). In addition, the expression of fibrosis inducers, such as interleukin *(IL)-4* and *IL-13*, could be suppressed by blocking the down-stream signaling pathways of *CCL3/CCL2* (8,9). These reports verified the significance of chemokines in fibrosis. The *C-X-C* chemokine receptor *(CXCR)4* is the receptor of *CXCL12 (SDF1)* and the main function of *CXCL12/CXCR4* is to regulate the transport of hematopoietic cells and secondary lymphoid tissue structures (10), which play an important role in the growth and development of renal vessels (11). A study has shown that *CXCR4* is a potential target for preventing renal fibrosis in patients with nephrolithiasis (12). Under renal ischemia, the expression of *CXCR4* is elevated (13). Recently, *CXCL12/CXCR4* was shown to promote the progression of renal fibrosis in unilateral ureteral ligated mice and rats with chronic transplanted kidney disease (14,15). However, the impact of *CXCR4* on renal fibrosis has only been reported in animal experiments, which lacked fundamental investigations on a cellular level. Moreover, renal fibrosis is a common pathophysiological change after the end-stage of chronic kidney disease. Inflammation also plays an important role in renal fibrosis, but non-inflammatory factors (such as ischemia and hypoxia) may also lead to renal fibrosis in the course of renal fibrosis. Therefore, we explore the effect of *CXCR4* gene on renal fibrosis from the perspective of non-inflammation. Apart from blood cells and immune cells, the glomeruli consists of glomerular basement membrane, mesangial matrix, glomerular endothelial cells, mesangial cells, and podocytes. Podocytes are targets of inflammatory and non-inflammatory injury in glomerular diseases, when podocytes are stimulated by various pathologies, it leads to the fusion and disappearance of podocytes and the abnormal number, shape and density of podocytes. The changes of pore membrane and cytoskeleton proteins destroy the integrity of glomerular filtration barrier and function, and cause mesangial cell proliferation and extracellular matrix metabolism disorder, resulting in glomerulosclerosis. The present study suppressed the activity of *CXCR4* in the glomerular endothelial cells, mesangial cells, and podocytes by transfecting a short hairpin (sh)RNA or utilizing the *CXCR4* inhibitor, to explore the impacts of *CXCR4* suppression on the expression of fibrosis-related genes. This investigation provided novel insights into the regulatory properties of *CXCR4* in glomerular cells under a non-inflammatory state. We present the following article in accordance with the MDAR and ARRIVE reporting checklists (available at https://tp.amegroups.com/article/view/10.21037/tp-22-157/rc).

Methods

Isolation and culture of primary glomerular endothelial cells, mesangial cells, and podocytes

A protocol was prepared before the study without registration. Animal experiments were performed under a project license (No. 2020052701) granted by ethics committee of Jiangxi Provincial Children's Hospital, in compliance with Jiangxi Provincial Children's Hospital institutional guidelines for the care and use of animals.

Mouse were sacrificed and the kidneys were isolated and placed into Hank's medium containing penicillin and streptomycin. Mouse purchased from Hunan slake Jingda experimental animal Co., Ltd. [License No: SCXK(Xiang)2021-0002], age 26-30 days. The kidney capsule was removed and the renal cortex was clipped into pieces. The glomerular endothelial cells were digested using 0.1% collagenase II solution, and the mesangial cells and podocytes were digested with 0.1% collagenase IV solution. The digestion steps were terminated by addition of complete medium. The digested tissues were percolated through a 100-μm filter, followed by centrifugation at 1,000 rpm for 5 minutes. Cells were collected and cultured using the corresponding medium. The culture medium for glomerular endothelial cells was DF12 containing 20% fetal bovine serum (FBS), penicillin, streptomycin, and vascular endothelial growth factor (*VEGF*). Mesangial cells were cultured in R1640 medium supplemented with 15% FBS, penicillin, streptomycin, and ITS (100×). Podocytes were cultured in KI medium and 3T3 medium at a ratio of 1:1. The KI medium contained DF12, HEPES (10 mM), penicillin, streptomycin, and ITS (100×). The 3T3 medium contained DMEM, 20% FBS, HEPES (10 mM), penicillin, streptomycin, glutamine (100×), and sodium pyruvate (1 mM).

Immunofluorescence assay

Treated cells were washed with phosphate buffered saline

Table 1 The sequences of primers for CXCR4 and GAPDH

Primer ID	Sequences	Length of the primer (bp)	Length of the product (bp)	Annealing temperature (℃)
CXCR4 F	GGGGTCATCAAGCAAGG	17	90	56.7
CXCR4 R	CAGGCAACAGTGGAAGAAGG	20		
GAPDH F	TCAACGGCACAGTCAAGG	18	357	57.8
GAPDH R	TGAGCCCTTCCACGATG	17		

CXCR4, *C-X-C* chemokine receptor type 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

(PBS) solution, fixed with 4% paraformaldehyde, and incubated with the following primary antibodies: rabbit anti-CD34, anti-desmin, anti-nephrin, and anti-WT-1. Cells were then incubation with the appropriate secondary antibody conjugated to DAPI (a blue nuclear marker). Negative control staining was performed by omitting the primary antibody. The stained cells were observed using a fluorescence microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells using TRIzol reagent (Thermo Fisher, Massachusetts, USA) according to the manufacturer's instructions. Transcription of cDNA from RNA using a SuperScript III kit (Takara, Tokyo, Japan). The relative expression of the detected proteins was detected using SYBR green quantitative PCR kit (Takara, Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the negative control. qRT-PCR was performed using the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to calculate and quantify the relative expression levels. The primer sequences used are shown in *Table 1*.

Transfection

The lentivirus containing the shRNA against *CXCR4* (sh-*CXCR4*) and the negative (blank) control (shNC) was purchased from Xiamen Lifeint Co., Ltd. The lentivirus was transfected into the cells with Lipofectamine 3000 to establish the *CXCR4* knockdown cell lines.

Western blot analysis

Cells were lysed with RIPA lysis buffer containing protease and phosphatase inhibitors, and then centrifuged at 14,000 rpm for 20 minutes at 4 ℃. The total concentration of proteins was detected using the BCA assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Approximately 80 μg of protein was isolated from each sample by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The samples were incubated with 5% skim milk for 90 minutes to remove the non-specific binding proteins. Membranes were then incubated with primary rabbit antibodies against *CXCR4*, *collagen IV*, *MMP-9*, *PI3K*, *Rac1*, *or VCAM-1* (1:1,000, Abcam, Massachusetts, USA) at 4 ℃ overnight. Subsequently washed with 0.1% Tris buffered saline Tween (TBST), the cell membranes were incubated with secondary antibodies and the immuno-reactive bands were observed by chemiluminescence using an ECL kit (Beyotime, Shanghai, China). The specific bands were analyzed using ImageJ software.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. All data are presented as mean ± standard deviation. Student's *t*-tests were used for inter-group comparisons. For comparisons of 3 or more groups, one-way analysis of variance (ANOVA) was conducted, followed by the Bonferroni post-hoc test. A P value <0.05 was considered statistically significant.

Results

The identification of primary glomerular endothelial cells, mesangial cells, and podocytes

Primary glomerular endothelial cells, mesangial cells, and podocytes were isolated and the purity was confirmed by immunofluorescence staining for *CD34*, desmin, and nephrin and *WT-1*, respectively (*Figure 1*). *CD34* was expressed on almost all the glomerular endothelial cells,

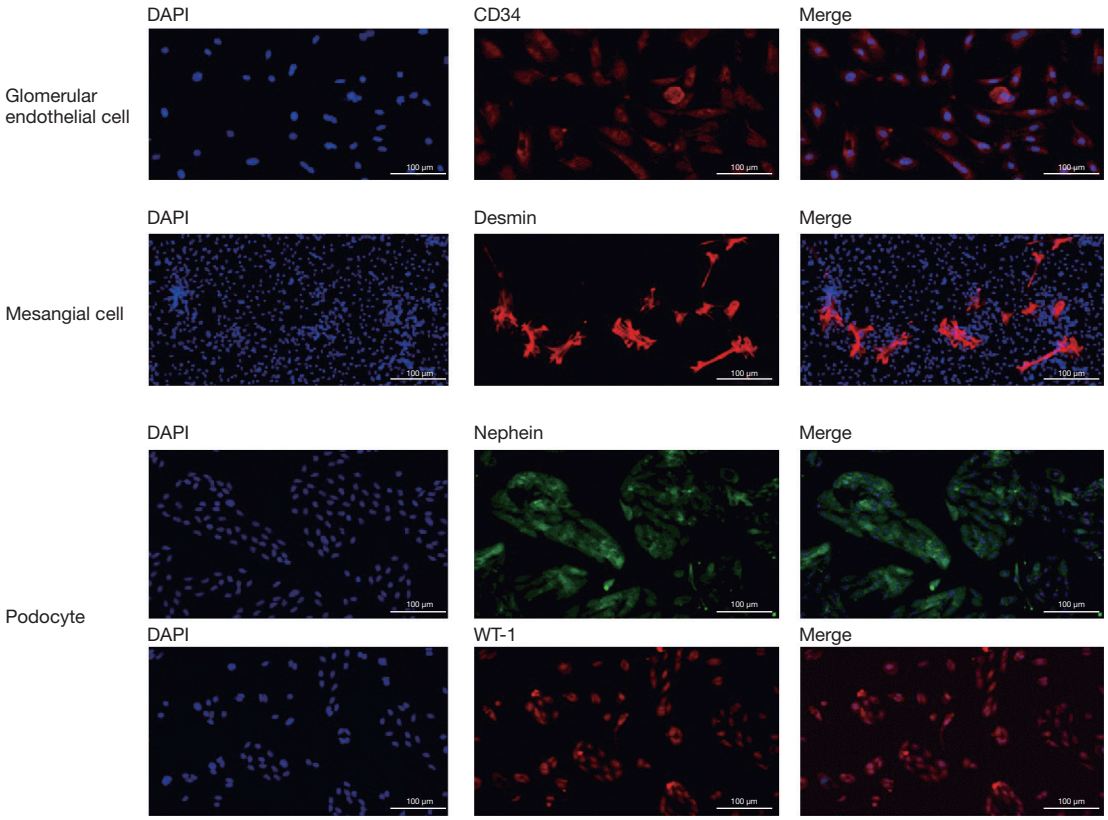

Figure 1 The isolated primary glomerular endothelial cells, mesangial cells, and podocytes were identified by immunofluorescence assays. Blue represents the nucleus dyed by DAPI. Red or green represents the target proteins.

and nephrin and *WT-1* were expressed on almost all the podocytes, indicating a relatively high purity for isolated primary glomerular endothelial cells and podocytes. In contrast, desmin was only expressed on a portion of the isolated mesangial cells, indicating relatively low purity for the isolated mesangial cells.

Suppressing the activity of CXCR4 downregulated the expression of renal fibrosis-related genes in primary glomerular cells

To confirm the transfection efficacy, qRT-PCR and Western blot assays were performed to assess the expression of *CXCR4*. As shown in *Figure 2*, compared to control cells, glomerular endothelial cells (HRGECs), mesangial cells (HRMCs), and podocytes (HRPs) transfected with the sh-*CXCR4* showed significantly suppressed expression of *CXCR4*, indicating a successful knockdown of *CXCR4* in all three cell lines by sh-*CXCR4* ($P<0.05$).

As shown in *Figure 3*, Western blot analyses demonstrated that the expression of *CXCR4*, *collagen IV*, *MMP-9*, *PI3K*, *Rac1*, and *VCAM-1* were dramatically downregulated in the cell lines transfected with sh-*CXCR4*. Furthermore, cell lines treated with T140, AMD3100, or LY294002 also showed markedly reduced expression of *CXCR4*, *collagen IV*, *MMP-9*, *PI3K*, *Rac1*, and *VCAM-1* compared to untreated cells. No significant differences were observed in the expression levels of these proteins between control cells and cells transfected with shNC ($P<0.05$).

Discussion

Tissue fibrosis is the process of repair following tissue injury, and if uncontrolled, this can contribute to sustained fibrosis which is characterized by destruction of the tissue structure and dysfunction of the organ (16). Renal fibrosis is the common denouement of various chronic renal diseases, which is mainly caused by the imbalance between the synthesis and the degradation of the extracellular matrix. Renal fibrosis is characterized by abnormal accumulation

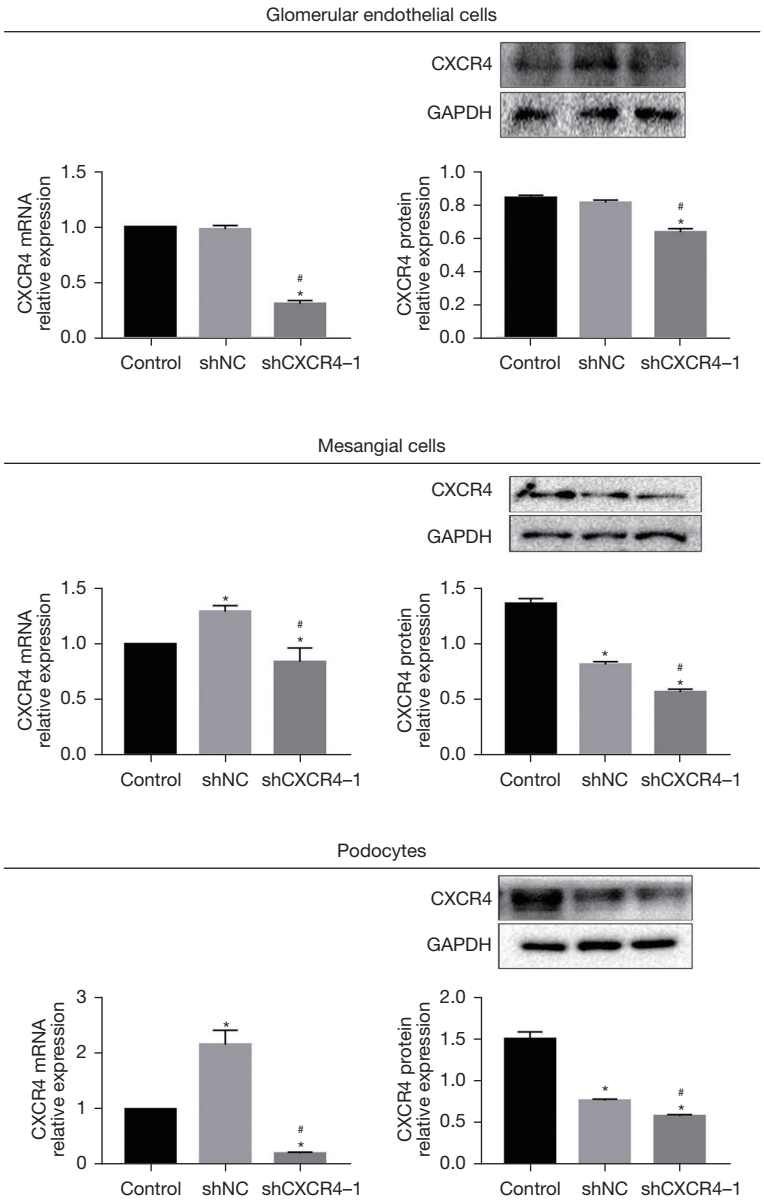

Figure 2 The expression of *CXCR4* in the glomerular endothelial cells, mesangial cells, and podocytes transfected with sh-*CXCR4* was evaluated by qRT-PCR and Western Blot assays. *, P<0.05, *vs.* control; #, P<0.05, sh*CXCR4-1 vs.* shNC. *CXCR4*, C-X-C chemokine receptor type 4; HRGEC, glomerular endothelial cells; HRMC, mesangial cells; HRP, podocytes; sh, short hairpin; qRT-PCR, quantitative real-time polymerase chain reaction; NC, negative control; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

of extracellular matrix, progressive changes in the structure of the kidney, and sustained dysfunction of the kidneys. To date, there are no effective radical therapies for the treatment of clinical renal fibrosis, with current therapies concentrating on delaying the progression of the disease. Therefore, understanding the molecular mechanisms underlying renal interstitial fibrosis is crucial for the development of potential targeted therapies to reverse renal interstitial fibrosis.

CXCR4 has been shown to play an important role in the pathological process of autoimmune diseases, including the fibrosis of the lungs and liver (17). Under the normal healthy state, *CXCR4* is expressed at relatively low levels in renal tissues. However, under the pathological state, such

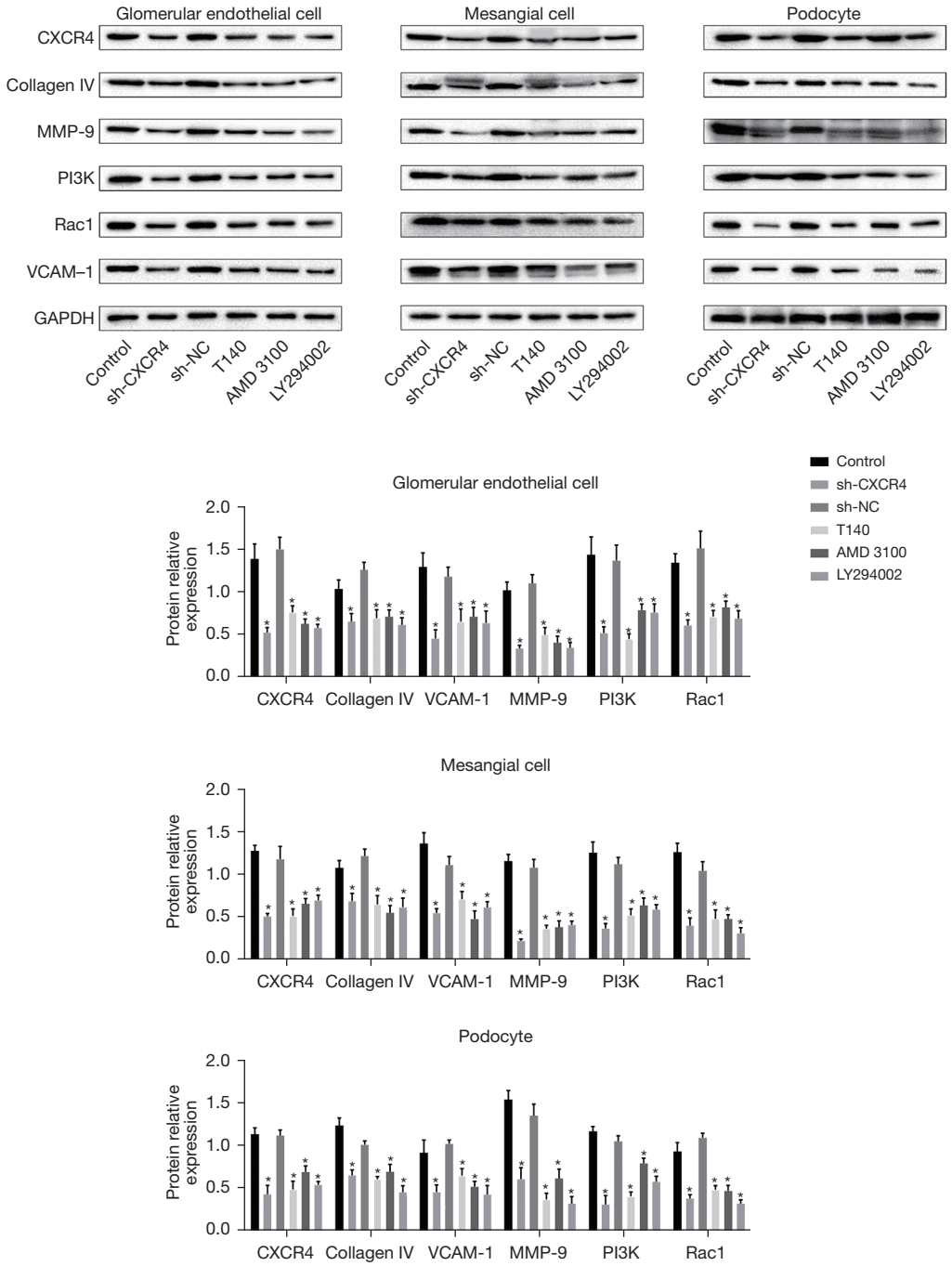

Figure 3 The expression of *CXCR4*, *Collagen IV*, *MMP-9*, *PI3K*, *Rac1*, and *VCAM-1* was evaluated by Western blot assays. *, P<0.05, *vs.* control. *CXCR4*, C-X-C chemokine receptor type 4; *MMP*, matrix metallopeptidase; *PI3K*, phosphoinositide 3-kinase; *Rac1*, Ras-related C3 botulinum toxin substrate 1; VCAM-1, vascular cell adhesion protein 1; sh, short hairpin; NC, negative control; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

as IgA nephropathy and focal stage glomerulosclerosis, the expression of *CXCR4* is significantly elevated (18). In addition, *CXCR4* has been shown to promote the chronic progression of renal fibrosis (14,19). In the present study, the expression levels of fibrosis-related genes (including *collagen IV*, *MMP-9*, and *VCAM-1*), *CXCR4*, and the

downstream regulatory elements (including *PI3K* and *Rac1*) were evaluated in the sh-*CXCR4* transfected isolated primary glomerular endothelial cells, mesangial cells, and podocytes to verify the function of *CXCR4* in the development and progression of renal fibrosis at a cellular level.

Collagen IV is the main component of glomerular basement membrane and constitutes the classical network structure in the kidney, together with laminin, proteoglycan, and paraplegic protein (20). Tissue repair and chronic inflammation can induce excessive accumulation of extracellular matrix components, such as collagen, which in turn, can be degraded by various MMPs. *MMP9* is a classic MMP involved in the development and progression of fibrosis. Although the progression of fibrosis is accompanied by upregulation of *MMP9*, both promoting and inhibitory effects have been reported for *MMP9* in the regulation of fibrosis (21,22). *VCAM-1* is involved in mediating the adhesion and signal transduction of leukocytes and is highly expressed in the kidneys. *VCAM-1* has also been shown to play an important role in the development of renal inflammation (23). In the present study, suppressing the activity of *CXCR4* in the isolated primary glomerular endothelial cells, mesangial cells, and podocytes by incubating with *CXCR4* inhibitors (T140 and AMD3100) significantly suppressed the expression of *collagen IV*, *MMP-9*, and *VCAM-1*. Similar results were observed when *PI3K* was inactivated by incubating cells with LY294002. A recent report has emphasized the positive correlation between *CXCR4* and the inflammatory reaction in the kidneys, and indicated that *CXCR4* inhibitors can ameliorate the symptoms of renal fibrosis by inhibiting renal inflammation through suppressing the recruitment of immune cells (24). In addition, it has also been reported that under the inflammatory state, *CXCR4* located on macrophages promoted the progression of renal fibrosis via various pathways. The recruitment of macrophages could be significantly suppressed by knocking down the expression of *CXCR4* on the macrophages, resulting in amelioration of renal fibrosis (25-27). In contrast to the glomerulus in the kidney, the isolated primary glomerular cells used in the present study were not influenced by other immune cells and inflammatory factors, and this is the main difference between the present study and previous reports. This investigation demonstrated that renal fibrosis-related genes could be regulated by *CXCR4* and the downstream signaling pathways in the glomerulus, even in the absence of inflammation.

PI3K can be activated by the Gβγ subunit bound by *CXCR4*, which further activates Rac2 to form the *Rac/Cdc42* complex, Finally, the chemotaxis reaction will be triggered (28). It has also been reported that *P-Rex1* can be activated by the Gβγ subunit, which activates Rac1 to bind GTP independently of *PI3K* (29). The present study showed that the expression of *PI3K* and *Rac1* was significantly suppressed in the primary glomerular endothelial cells, mesangial cells, and podocytes by knocking down or inactivating *CXCR4*, and this was similar to the effects observed with the *PI3K* inhibitor. We speculate that in the glomerulus, *PI3K/Rac* is the downstream signaling pathway of *CXCR4*, which regulates the expression of fibrosis-related genes.

The highest expression of *CXCR4* is normally observed in the bone marrow lymphatics and the circulatory system. It is also highly expressed in the kidneys, the gastrointestinal tract, and the lungs (30). T140, an inhibitor of *CXCR4*, has been shown to specifically inhibit *CXCR4* (31), and this may represent a promising targeted agent against *CXCR4*. The results of this investigation suggested that synergistic effects may be observed when combining inflammation inhibitory therapy with a *CXCR4* inhibitor, and this may be a novel potential therapeutic regimen for the treatment of renal fibrosis.

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Footnote

Reporting Checklist: The authors have completed the MDAR and ARRIVE reporting checklists. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-157/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-22-157/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-157/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2020052701) granted by ethics committee of Jiangxi Provincial Children's Hospital, in compliance with Jiangxi Provincial Children's Hospital institutional guidelines for the care and use of animals.

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